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FULL-LIFE CHRONIC TOXICITY OF SODIUM SALTS TO THE MAYFLY *NEOCLOEON TRIANGULIFER* IN TESTS WITH LABORATORY CULTURED FOOD

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Abstract: Although insects occur in nearly all freshwater ecosystems, few sensitive insect models exist for use in determining the toxicity of contaminants. The objectives of the present study were to adapt previously developed culturing and toxicity testing methods for the mayfly *Neocloeon triangulifer* (Ephemeroptera: Baetidae), and to further develop a method for chronic toxicity tests spanning organism ages of less than 24 h post hatch to adult emergence, using a laboratory cultured diatom diet. The authors conducted 96-h fed acute tests and full-life chronic toxicity tests with sodium chloride, sodium nitrate, and sodium sulfate. The authors generated 96-h median lethal concentrations (LC50s) of 1062 mg Cl/L (mean of 3 tests), 179 mg $N\text{-}NO_3$/L, and 1227 mg SO_4/L. Acute to chronic ratios ranged from 2.1 to 6.4 for chloride, 2.5 to 5.1 for nitrate, and 2.3 to 8.5 for sulfate. The endpoints related to survival and development time were consistently the most sensitive in the tests. The chronic values generated for chloride were in the same range as those generated by others using natural foods. Furthermore, our weight-versus-fecundity plots were similar to those previously published using the food culturing method on which the present authors' method was based, indicating good potential for standardization. The authors believe that the continued use of this sensitive mayfly species in laboratory studies will help to close the gap in understanding between standard laboratory toxicity test results and field-based observations of community impairment. *Environ Toxicol Chem* 2015;34:2126–2137. © 2015 SETAC

Keywords: Mayfly Toxicity testing Chronic toxicity Major ions

INTRODUCTION

Despite the fact that insects occur in nearly all freshwater ecosystems and are often the dominant class of macroinvertebrate in those systems [1], few insect models exist for use in determining the toxicity of contaminants. Among the most commonly used insect species in toxicity testing are the midges *Chironomus riparius* and *Chironomus dilutus*, which can be cultured in the laboratory but tend to be among the more insensitive invertebrates. Efforts have been made to assess the potential for use of abundant, wild-caught mayflies (Ephemeroptera) for standardized toxicity test development [2,3], but knowledge of exposure history, health, and age of test organisms before testing provides for better interlaboratory test result comparisons [4].

A major step toward the development of a sensitive insect model organism for toxicity testing was taken when Sweeney and Vannote [5] characterized the effects of several variables on life history characteristics of the parthenogenetic mayfly *Neocloeon triangulifer* McDunnough (originally described as *Cloeon triangulifer* [6], later transferred to *Centroptilum* [7], and most recently assigned to *Neocloeon* [8]). The same research group (Stroud Water Research Center) later developed a method of culturing this organism in the laboratory and using it in toxicity tests [9]. The advantage of *N. triangulifer* is that because it is parthenogenetic, it does not require a large amount of space for mating to take place; female clones can emerge into small containers (e.g., 300-mL beakers) and then deposit viable eggs. Furthermore, parthenogenic organisms in general are desirable for use in toxicity testing because being clonal eliminates genetic variability as a confounding factor. The Stroud Water Research Center group uses naturally colonized periphyton plates to feed the organisms, and this approach has now been adapted by others to conduct toxicological investigations with various contaminants [10–13].

Because food quality and quantity appear to influence the response of *N. triangulifer* to contaminants [10,11,13], developing a cultured food consisting of 1 or more diatom species is necessary to further allow standardization and more widespread use of this organism in toxicity testing. Great progress has been made toward this end by Weaver et al. [14], who developed a method of culturing 3 different diatom species (*Mayamea atomus, Nitzschia* cf. *pusilla,* and *Achnanthidium minutissimum*) in the laboratory, and then using stocks of monocultures of the species to colonize microscope slides, which are then offered to nymphs as biofilms. This approach was successfully used to culture 13 successive generations of the organisms, and both acute and short-term chronic (i.e., less than full-life) toxicity data have been generated using organisms cultured with this food [15].

Previously reported chronic toxicity tests with this species (or genus) have been conducted using wild-caught individuals [16], using cultured individuals with naturally colonized periphyton plates ([9–13,17,18]; J. Jackson, Stroud Water Research Center, Avondale, PA, USA, unpublished data), or for less than a full life cycle [15,19]. The objectives of the present study were to adapt the previously developed diatom culturing and toxicity testing methods for *N. triangulifer* [14,15] and to further develop a method for chronic toxicity tests spanning organism ages of less than 24 h post hatch to adult emergence, with the goal of producing data that are suitable for use in the development of chronic water quality criteria, as prescribed by Stephan et al. [20] (i.e., similar in scope to the daphnid and mysid life-cycle tests). We chose 3 sodium salts that are contaminants of interest in the Great Lakes region: sodium chloride, sodium nitrate, and sodium sulfate (E. Hammer, United States Environmental Protection Agency [USEPA],

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Washington, DC, USA, personal communication), and conducted 96-h fed acute tests and full-life chronic toxicity tests so that acute-to-chronic ratios could be calculated.

METHODS

Culturing of test organisms and food

Mayfly and diatom biofilm culturing methods were based on those developed by Weaver et al. [14], with several modifications. As recommended [14], we initially used 3 species of diatom for feeding of mayflies, but we stopped using *Achnanthidium* sp. because it was more difficult to culture than the other diatoms, and culture results indicated that it was not necessary (DJ Soucek, personal observation). Diatoms used to feed mayflies included *Mayamea* sp. and *Nitzschia* sp. Both diatoms were obtained from Carolina Biological Supply, sold as *Navicula* sp. and *Synedra* sp., respectively. We had the genus-level identities taxonomically confirmed by an expert (S. Decelles) at USEPA-ORD, Cincinnati, Ohio, USA.

Mixed diatom stocks

To culture diatoms, we autoclaved (30 min at 121 °C, liquid cycle) a 4-L flask containing 4 L filtered (Whatman 934-AH) dechlorinated tap water and a 2-inch-long, Teflon-coated stir bar. After allowing it to cool, sterile technique was used to add 1.3 mL Kent Proculture Professional F/2 Algal culture formula A, 1.3 mL Kent Proculture Professional F/2 Algal culture formula B, 150 mg sodium metasilicate ($Na_2SiO_3 \cdot 9H_2O$), and 200 mL fresh diatom stock solution (just removed from stir-plate). Both diatom species were present in combination in stock cultures. We had initially kept the species stocks separate, but combining the species in stocks did not appear to impact the performance of the mayflies, so we adopted the combined stock approach. The flasks were placed on stir-plates with moderate to fast stirring (a large vortex was visible) in an environmental chamber set for a 16:8-h light:dark photoperiod and 25 °C. Diatom stocks were allowed to grow for 5 d, then 200 mL stock was used to seed the next flask and cages for mixed diatom slides (see below *Mixed-diatom slides*). Stocks were not refrigerated before seeding subsequent flasks or mixed-diatom slide cages.

Mixed-diatom slides

To culture mixed-diatom slides, 15 fully frosted microscope slides (catalogue no. 12-544-5CY, Fisherscience) were placed in a single layer (with frosted side facing up) on the bottom of a 7.2-L (189 mm × 297 mm × 128 mm) autoclavable polysulfone mouse cage (#PC7115HT, Ace Caging) filled with 2.5 L filtered (Whatman 934-AH) dechlorinated tap water. The container with the slides was autoclaved (30 min at 121 °C, liquid cycle) and allowed to cool. Sterile technique was used to add 1.3 mL Kent Proculture Professional F/2 Algal culture formula A, 1.3 mL Kent Proculture Professional F/2 Algal culture formula B, 150 mg sodium metasilicate ($Na_2SiO_3 \cdot 9H_2O$; dissolved in a small amount of deionized water before addition), and 200 mL fresh (never refrigerated) mixed-diatom stock. The container with slides was covered with clear plastic wrap and placed in an environmental chamber set for a 16:8-h light:dark photoperiod and 25 °C. Slides tended to have the most robust biofilms if used within approximately 6 d to 10 d after inoculation, but slides as old as 1 mo were used with success as long as most of the material appeared to be distinct round or spindle-shaped diatom cells when examined under a compound microscope. Poor-quality slides would have very few distinct cells on microscopic examination and would be mostly masses of amorphous material. If, after approximately 5 d, the biofilms appeared to be thin, we mildly aerated cages containing slides rather than adding more nutrients. We made the observation that color of the biofilm slides, as apparent to the naked eye, was not necessarily predictive of good mayfly performance. Therefore, before feeding to mayflies in toxicity tests or cultures, diatom biofilms were examined under a compound microscope to ensure that most of the biofilm material was distinct diatom cells, rather than being amorphous material.

Mayfly nymph rearing method

Mayflies (*Neocloeon triangulifer*; Stroud Water Research Center Clone #WCC-2) were reared in an environmental chamber at 25 °C, and a 16:8-h light:dark photoperiod. Culture water was a reconstituted water (hereafter referred to as Duluth 100) with a nominal hardness of 100 mg/L as $CaCO_3$, prepared according to a formula developed at the USEPA laboratory in Duluth, Minnesota, USA. To make this water, the following salt concentrations were added to deionized water from a Barnstead "E-pure" filtration system: $KHCO_3$, 10 mg/L; $NaHCO_3$, 125 mg/L; $MgSO_4$, 38 mg/L; $CaSO_4$, 40 mg/L; $CaCl_2$, 43 mg/L; NaBr, 0.05 mg/L. This water recipe was designed with the goal of better mimicking chemistry of "typical" North American freshwaters relative to other commonly used reconstituted waters (D. Mount, USEPA, Washington, DC, USA, personal communication). When eggs hatched, approximately 250 mL culture water was added to a 300-mL "I-chem" jar. All water was filtered using Whatman #934-AH glass microfiber filters. One mixed diatom slide was added to the jar. Newly hatched mayfly larvae (100–1000s) were then added to the jar, the lid was loosely placed, and the jar was covered with aluminum foil to block direct overhead lighting. When mayflies were 4 d to 8 d old (usually 6 d or 7 d), 40 individuals were placed in a 1-L beaker containing 400 mL Duluth 100 reconstituted water, and fed as described for the I-chem jar. The diatom slide was placed in the beaker before adding mayflies to avoid injury. Again, the container was covered with aluminum foil to block direct overhead lighting. When mayflies were 11 d to 12 d old, 20 individuals were transferred to a 19-cm × 24-cm × 6.5-cm Pyrex casserole dish containing 1.5 L Duluth 100 water and 5 mixed diatom slides. Slides were replaced when diatom biofilms were depleted, and water was changed twice per week or more if water appeared to be littered with loose diatoms and waste products. The container was covered loosely with foil. Using this method, aeration was not necessary at any point during mayfly culturing.

When pre-emergent nymph stages (determined by presence of black wing pads) appeared (days 20–23), they were placed in a 300-mL I-chem jar containing culture water and a mixed diatom slide. A screened cover was placed on the jar to allow for emergence of sub-imagoes and molting to imago stage (within 24 h after pre-emergent nymph stage). To induce the imago to release its eggs, we held it by the wings with forceps and touched its abdomen to culture water held in a small petri dish. This procedure was conducted with the aid of a dissecting microscope. Eggs were then pipetted into a scintillation vial; when possible, eggs of 3 females were combined in each vial. Eggs were either allowed to hatch or placed in an environmental chamber at 10 °C for later use. We observed a predictable relationship between the number of days eggs were held at 10 °C and the number of days to hatch on transfer of eggs to 25 °C (Figure 1). In some cases, eggs hatched over multiple days. For the purposes of generating a predictive equation for time to egg hatch, we used half-day intervals; for

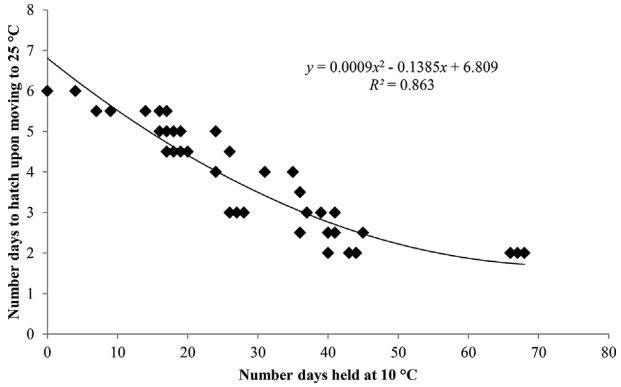

Figure 1. Predictive relationship between the number of days *Neocloeon triangulifer* eggs are held at 10 °C and number of days to hatch on moving eggs to 25 °C.

example, if approximately equal numbers of eggs hatched on days 2 and 3 after placement in 25 °C, we used 2.5 d in the regression model. The predictive model is as follows: # days to hatch on moving eggs to 25 °C = 0.0009 (# days at 10 °C)^2 – 0.1385(# days at 10 °C) + 6.809.

Test chemicals and dilution water

The nitrate, chloride, and sulfate sources for acute and chronic toxicity tests were reagent-grade sodium salts ($NaNO_3$ CAS # 7631-99-4; NaCl CAS # 7647-14-5; Na_2SO_4 CAS # 777-82-6). All acute and chronic tests were conducted in Duluth 100 hard water. As noted in the section *Mayfly nymph rearing method*, this was also the mayfly culture water, and eggs were stored in this water, so no acclimation was required.

Starvation test

To confirm previous observations that young mayflies are unable to survive extended periods without food [15], we conducted a starvation experiment in which we placed 1 <24-h-old mayfly into each of twenty 30-mL beakers containing 20 mL Duluth 100 water at 25 °C. We added no food to the beakers and observed mortality over the next 48 h. All organisms were alive at 24 h, but by the next day (48 h), survival was down to 22% (Figure 2). Based on this finding, all acute toxicity tests were fed a scraping of mixed diatom biofilm (described in *Acute test procedures*). Because all tests were conducted with sodium salts, food was not expected to impact availability of the contaminants, and analytical chemistry confirmed this (described in *Acute test procedures*).

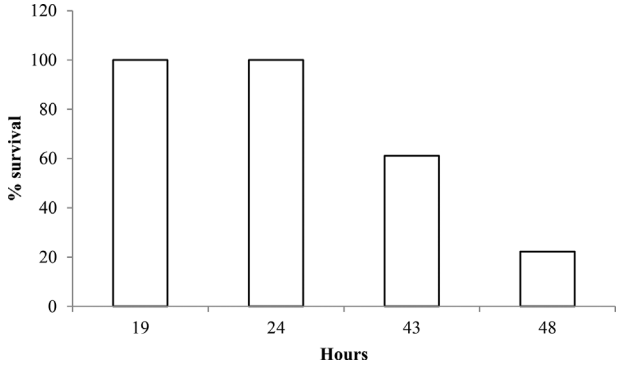

Figure 2. Percentage of survival of *Neocloeon triangulifer* larvae over time with no food. Organisms were less than 24 h old at the start of the test.

Table 1. Test conditions for acute toxicity tests with *Neocloeon triangulifer*

Condition	Value
1. Temperature (°C)	25 ± 1
2. Photoperiod (light:dark)	16:8
3. Test chamber size	30 mL
4. Test solution volume	20 mL
5. Age of organisms	<24 h
6. Dilution water	Duluth 100
7. Substrate	None
8. No. of organisms per chamber	5
9. No. of chambers/treatment	4
10. Food	Scraping of live diatom biofilm
11. Aeration	None
12. Test type	Static
13. Renewal frequency	None
14. Test duration	96 h
15. Control survival	≥90%
15. Endpoint	Survival

Acute test procedures

Static, nonrenewal, acute toxicity tests were conducted according to guidelines detailed in ASTM International E729-96 [4]. Treatments comprised a 50% dilution series. Five concentrations were tested in addition to controls. Further general details on test conditions are provided in Table 1. Organisms were less than 24 h old at the beginning of the test. Test chambers were fed by grasping a mixed diatom slide (cultured as described previously in *Mixed-diatom slides*) with a forceps, and scraping off an approximately 5-mm × 10-mm × 25-mm area of biofilm with another clean microscope slide, and releasing the biofilm into the test chamber. Chambers were fed on day 0 only, because 1 biofilm scraping was more than enough for the 96-h test duration. Mortality was assessed daily, using a dissecting microscope. Individuals were considered dead if they did not respond to gentle prodding with a blunt instrument. All median lethal concentration (LC50) values were calculated by using the trimmed Spearman-Karber method [21].

Standard water chemistry parameters were measured at both the beginning and the end of each exposure period, including temperature, pH, conductivity, and dissolved oxygen. Alkalinity and hardness were measured at the beginning of the test only. The pH measurements were made by using an Accumet (Fisher Scientific) model AB15 pH meter equipped with an Accumet gel-filled combination electrode (accuracy < ±0.05 pH at 25 °C). Dissolved oxygen was measured using an air-calibrated Yellow Springs Instruments (RDP) model 55 meter. Conductivity measurements were made using a Mettler Toledo (Fisher Scientific) model MC226 conductivity/total dissolved solids meter. Alkalinity and hardness were measured by titration [22]. Water samples from each treatment were collected at the beginning and end of acute tests and submitted to the Illinois State Water Survey analytical laboratory for measurement of nitrate, chloride, and sulfate concentrations as appropriate, using ion chromatography. Conductivity varied with salt concentration in all 3 tests. For the NaCl tests, mean (±standard deviation [SD]) temperature, pH, dissolved oxygen, alkalinity, and hardness were 25.0 ± 0.2 °C, 8.3 ± 0.1, 7.5 ± 0.3 mg/L, 84 ± 2 mg/L as $CaCO_3$, and 93 ± 1 mg/L as $CaCO_3$, respectively. For the $NaNO_3$ test, the values were 24.7 ± 0.3 °C, 8.3 ± 0.1, 7.8 ± 0.1 mg/L, 83 ± 1 mg/L as $CaCO_3$, and 99 ± 1 mg/L as $CaCO_3$, respectively. For the Na_2SO_4 test, the values were 24.8 ± 0.2 °C, 8.3 ± 0.1, 7.6 ± 0.2 mg/L, 84 ± 2 mg/L as $CaCO_3$, and 99 ± 1 mg/L as $CaCO_3$, respectively. For the NaCl test, measured chloride concentrations averaged 103%

of nominal (range, 100–106%); for the $NaNO_3$ test, measured N-NO_3 averaged 105% of nominal (range, 103–107%); for the Na_2SO_4 test, measured sulfate averaged 107% of nominal (range, 101–110%). All reported LC50 values are based on measured concentrations.

Chronic toxicity testing procedures

For chronic toxicity tests, 6 treatments, including a control (dilution water), were tested. Nominal chloride concentrations for the treatments were as follows: 37 mg Cl^-/L (control), 70 mg Cl^-/L, 112 mg Cl^-/L, 196 mg Cl^-/L, 364 mg Cl^-/L, and 700 mg Cl^-/L. Nominal N-NO_3 concentrations were as follows: 0 mg N-NO_3/L (control), 12.5 mg N-NO_3/L, 25 mg N-NO_3/L, 50 mg N-NO_3/L, 100 mg N-NO_3/L, and 200 mg N-NO_3/L. Nominal SO_4^{2-} concentrations were as follows: 59 mg SO_4^{2-}/L (control), 136 mg SO_4^{2-}/L, 214 mg SO_4^{2-}/L, 369 mg SO_4^{2-}/L, 679 mg SO_4^{2-}/L, and 1300 mg SO_4^{2-}/L.

Test conditions are summarized in Table 2. In an attempt to minimize the mass of diatoms required for each test chamber, we initially used the model of the ASTM International *Ceriodaphnia dubia* chronic method [23], in which 1 organism is added per test chamber, and each treatment has 10 replicates. The NaCl and $NaNO_3$ chronic tests were conducted in this manner, but for the Na_2SO_4 chronic test, we used 2 organisms per test chamber (a total of 20 organisms per treatment) to decrease the chance of random control mortalities causing test failure. Because having 2 individuals per chamber did not require substantially higher numbers of diatom slides through the course of the test, we recommend using this organism loading rate for further tests.

Before the start of a test, a vial containing eggs from 3 females was moved from the 10 °C environmental chamber to the 25 °C chamber to encourage hatching of the eggs. The test began when sufficient numbers hatched to conduct the test; organisms were less than 24 h old at the start. Tests were conducted at 25 ± 1 °C and a 16:8-h light:dark photoperiod. All test chambers were covered with plastic wrap to minimize evaporation and with aluminum foil to eliminate direct overhead light. Light intensity in test chambers was approximately 110 lux to 160 lux. The test chambers at the beginning of the test were 30-mL glass beakers with 20 mL test water (or control). No substrate was used, but a scraping from a mixed diatom biofilm slide was added to each beaker for food as described previously in *Acute test procedures* for acute testing. Chambers were fed on day 0 and day 5, then on every water change day thereafter. Beginning on day 17 and until the end of the tests, biofilm scrapings were added to test chambers daily. Beakers were never allowed to be empty of food before the next feeding. We used scrapings of live biofilms for food, because numerous previous experiments using concentrated, refrigerated mixed diatom suspension made from concentrated stocks of freshwater diatoms, as recommended by Struewing et al. [15], resulted in lack of development of nymphs (DJ Soucek, personal observation). Water was not renewed for the first 4 d of the test, because the small size of the test organisms at that time made handling a potential source of error. Beginning on day 5 and every Monday, Wednesday, and Friday thereafter, complete water changes were conducted, with organisms being transferred to new beakers with freshly made water. Organisms were held in 30-mL beakers until day 14, when they were moved to 150-mL glass beakers with 100 mL test water. The 150-mL beakers were used until just before emergence.

Mayflies began to reach the pre-emergent nymph stage on day 20 to day 22, depending on the test. Slower developing individuals took as many as 36 d to reach this stage, but most had done so by day 25 in all 3 tests. When pre-emergent nymph stages were observed, they were transferred to 300-mL glass beakers with 125 mL appropriate test or control water and food. In early experiments to test this method, we added a clean microscope slide placed at a slant in the beaker to provide a surface for organisms to walk up and exit the water surface, but we later learned that this was not necessary. The larger beakers were used to provide more space for the flying sub-imagoes/imagoes, and they were covered individually with plastic wrap to prevent escape of the organisms. Sub-imagoes and imagoes were able to rest by clinging to the beaker wall or plastic wrap cover. For the NaCl and $NaNO_3$ tests, each organism had its own emergence chamber. In the case of the Na_2SO_4 chronic, which had 2 organisms per chamber at the beginning of the test, if both individuals reached pre-emergent nymph stage on the same day, they were placed in the same emergence chamber. Otherwise, each individual had its own emergence chamber. The pre-emergent nymph-staged nymphs were observed in the morning, and by the next morning, if successful, they had molted through the sub-imago stage to the imago stage. Imagoes then were grasped by the wings with a forceps, and weighed live to the nearest 0.001 mg by using a Cahn C-35 microbalance. Then, holding them again by the wings using a forceps, we held the ventral surface of their abdomen to Duluth 100 water held in a thick depression slide (85 mm × 14 mm × 33 mm), allowing the adult to release its eggs. When this procedure is performed under a dissecting scope, a faint yellowish coloration formed by the eggs is visible in the side of the abdomen. When all of the eggs are released, this coloration is no longer visible. In addition, we watched through the scope as the eggs were released and continued to hold the abdomen to the water for some time after eggs stopped appearing, to ensure that all eggs

Table 2. Test conditions for chronic toxicity tests with *Neocloeon triangulifer*

Condition	Value
1. Temperature (°C)	25 ± 1
2. Photoperiod (light: dark)	16:8
3. Test chamber size/solution volume	Day 0–14: 30 mL/20 mL; day 14 pre-emergent nymph stage: 150 mL/100 mL; emergence chamber: 300 mL/125 mL
4. Age of organisms at start of test	<24 h
5. Dilution water	Duluth 100
6. Substrate	None
7. No. of organisms per chamber	2 (2 of our tests had 1 per chamber)
8. No. of chambers/treatment	10
9. Food	Scrapings of live diatom biofilms from slides Day 0–16 on water change days; thereafter, daily
10. Aeration	None
11. Test type	Static/renewal
12. Renewal frequency	Days 0–4: none; day 5—end of test: MWF
13. Control survival[a]	≥80%
14. Endpoints	%Survival to pre-emergent nymph, % pre-emergent nymph when controls finished, no. of days to pre-emergent nymph, % emergence, pre-egg laying wet weight of adult, no. of eggs/original female

[a]Control survival was evaluated as no. of organisms surviving to pre-emergent nymph stage.
MWF = Monday, Wednesday, Friday.

were released. With the eggs in the depression slide, we used a dissection probe to break the surface tension, allowing the eggs to fall to the bottom of the depression. The concave nature of the depression caused eggs to gather in the bottom. We then used the probe to spread the eggs into a monolayer, and photographed the monolayer using an Olympus Q-color 3 camera mounted on a dissecting scope and QCapture Ver 2.7.3 software. The images were then printed to hard copy, and eggs were counted manually.

Standard water chemistry parameters were measured throughout the exposure period, including temperature, pH, conductivity, dissolved oxygen, alkalinity, and hardness, as detailed previously in *Acute test procedures* for acute toxicity tests. Water samples from each treatment were submitted to the Illinois State Water Survey analytical laboratory for confirmation of chloride, sulfate, or nitrate concentrations by using ion chromatography. With the exception of conductivity, which varied with salt concentration in all 3 tests, measured water quality parameters varied little in the chronic toxicity tests. For the NaCl test, mean (±SD) temperature, pH, dissolved oxygen, alkalinity, and hardness were 25.2 ± 0.5 °C, 8.3 ± 0.1 mg/L, 7.4 ± 0.4 (lowest value = 6.8) mg/L, 86 ± 4 mg/L as $CaCO_3$, and 94 ± 3 mg/L as $CaCO_3$, respectively. For the $NaNO_3$ test, the values were 25.2 ± 0.3 °C, 8.3 ± 0.1 mg/L, 7.5 ± 0.5 (lowest value = 6.6) mg/L, 85 ± 5 mg/L as $CaCO_3$, and 95 ± 7 mg/L as $CaCO_3$, respectively. For the Na_2SO_4 test, the values were 25.0 ± 0.3 °C, 8.4 ± 0.1 mg/L, 7.3 ± 0.4 (lowest value = 6.1) mg/L, 83 ± 3 mg/L as $CaCO_3$, and 95 ± 4 mg/L as $CaCO_3$, respectively. For the NaCl test, measured chloride concentrations averaged 100% of nominal (range, 92–109%); for the $NaNO_3$ test, measured N-NO_3 averaged 102% of nominal (range, 96–107%); for the Na_2SO_4 test, measured sulfate averaged 97% of nominal (range, 90–106%).

Endpoints measured included percentage of survival to pre-emergent nymph stage; percentage of pre-emergent nymph when controls finished (% pre-emergent nymph when controls finished, calculated as the number of individuals in a treatment that had successfully reached pre-emergent nymph stage by the day the last individual in the control reached pre-emergent nymph stage); mean number of days until pre-emergent nymph stage; percentage of emergence (calculated as the number successfully emerging to imago stage divided by the number of individuals at the start of the test), mean pre-egg-laying wet (live) weight of imago; number of eggs per female (i.e., number of eggs per emerging female, a measure of mean individual fecundity); number of eggs per original female (i.e., overall number of eggs produced in a treatment divided by the number of individuals at the start of the test). The latter endpoint is analogous to population growth rate, which would include development time as well, but because the results of the 2 calculations were highly correlated, we only included number of eggs per original female in the present study. Another endpoint reported by the Stroud Water Research Center (J. Jackson, Stroud Water Research Center, Avondale, PA, USA, unpublished data) was instantaneous growth rate, which incorporates adult and hatchling mass and development time. This endpoint was not responsive to any of the salts tested in the present study, so for the sake of brevity, we have not included it. Percentage of survival was analyzed statistically by using Fisher's exact test, and means for the remaining endpoints except for percentage of emergence and number of eggs per original female were compared by using analysis of variance with post hoc pairwise comparisons conducted using Tukey's honest significant difference. Dunnett's test was not used for post hoc tests for sublethal endpoints, because the treatments did not have equal replication because of mortality. The maximum allowable toxicant concentration was calculated as the geometric mean of the no observable effect concentration (NOEC: the highest concentration at which means were not significantly different from the control) and the least observable effect concentration: the lowest concentration at which means were significantly different from that of the control). When possible, 20% effective concentrations (EC20s) were calculated by using TRAP software (Ver 1.21A; R.J. Erickson, USEPA, MED, Duluth, MN, USA).

RESULTS

Acute toxicity tests

In the five 96-h, fed, acute toxicity tests conducted with *Neocloeon triangulifer* (3 with sodium chloride and 1 each with sodium sulfate and sodium nitrate), control survival was 100% in all cases except for the sodium sulfate test, which had 95% control survival. For the sodium chloride tests, individual 96-h LC50s (95% confidence intervals) were 1140 (994–1309) mg Cl/L, 910 (719–1153) mg Cl/L, and 1153 (96–1374) mg Cl/L. The geometric mean of these 3 tests (1062 mg Cl/L) was used to calculate the acute-to-chronic ratios for chloride. The 96-h LC50 for the nitrate test was 179 (165–205) mg N-NO_3/L, and the value for the sulfate test was 1227 (1073–1404) mg SO_4/L.

Chronic toxicity tests

Chloride test. Survival to pre-emergent nymph stage was high for the controls and the 3 lowest-exposure treatments (Table 3). In the 194-mg/L treatment, 1 individual was killed because of technician error, so survival was 8 of 9 individuals/replicates. The 362-mg/L and 701-mg/L treatments had significantly lower survival to pre-emergent nymph stage than the other treatments. All of the organisms in the 701-mg/L treatment were dead by day 4, and all mortality in the 362-mg/L treatment occurred on day 7 or before (Figure 3A). Percentage of pre-emergent nymph when controls finished was slightly more sensitive than the percentage of survival to pre-emergent nymph stage (20% vs 60%, respectively, in the 362 mg Cl^-/L treatment), but calculated effects levels were similar in the 2 endpoints. Controls reached the pre-emergent nymph stage by day 23 on average, as did the 70-mg/L, 112-mg/L, and 194-mg/L treatments. Organisms that survived in the 362-mg/L treatment had significantly delayed (by ~3 d) development to pre-emergent nymph stage. The percentage of emergence was relatively high in the controls, but although the highest concentration had no emergence, no dose–response relationship was observed for this endpoint. The measured maximum allowable toxicant concentration and EC20 (95% confidence interval) for percentage of pre-emergent nymph when controls finished were 265 mg Cl^-/L and 165 mg Cl^-/L (119–230 mg Cl^-/L), respectively, and for percentage of survival to pre-emergent nymph stage were 265 mg Cl^-/L and 190 mg Cl^-/L (129–280 mg Cl^-/L), respectively. We could not calculate an EC20 for number of days to pre-emergent nymph stage (as 1/mean number of days) or percentage of emergence because of insufficient slope, but the maximum allowable toxicant concentrations were 265 mg Cl^-/L and 504 mg Cl^-/L, respectively.

The sublethal endpoints of pre-egg-laying weight, number of eggs per female, and number of eggs per original female were not as sensitive as the survival-related endpoints. In fact, mean weights and number of eggs for the surviving individuals in the 362-mg Cl^-/L treatment were nominally higher than those for

Table 3. Chronic chloride (as NaCl) toxicity data for the mayfly *Neocloeon triangulifer*[a]

$[Cl^-]$[b] (mg/L)	Conductivity[c] (μmhos/cm)	% pre-emergent nymph WCF[d]	% survival to pre-emergent nymph stage	No. of days to pre-emergent nymph	% emergence	Pre-egg laying weight (mg)	No. of eggs per female	No. of eggs per original female
27	371 ± 11	100 A	100 A	23.3 ± 1.1 A	70 A	3.131 ± 0.311 A	1446 ± 146 A	964
70	516 ± 15	100 A	100 A	23.1 ± 0.7 A	50 A	2.949 ± 0.290 A	1302 ± 216 A	651
112	658 ± 15	90 A	90 A	23.1 ± 0.6 A	40 A	3.075 ± 0.293 A	1326 ± 107 A	531
194	939 ± 11	89 A	89 A	23.0 ± 0.5 A	78 A	2.996 ± 0.145 A	1206 ± 163 A	938
362	1479 ± 33	20 B	60 B	26.5 ± 2.0 B	60 A	3.246 ± 0.179 A	1497 ± 113 A	898
701	2550 ($n = 1$)	0 B	0 B	NA	0 B	NA	NA	0
MATC (mg Cl/L)		265	265	265	504	NC	NC	NC
EC20 (mg Cl/L)		165	190	NC	NC	NC	NC	NC
EC20 95% CI		119–230	129–280	NC	NC	NC	NC	NC
ACR[e]		6.4	5.6	4.0	2.1	NC	NC	NC

[a]Within endpoint columns, means followed by different capital letters are significantly different ($p < 0.05$).
[b]Mean measured Cl^- concentrations are shown.
[c]Conductivity values shown are means (±Standard deviation) of all measurements for the duration of the test.
[d]WCF = when controls finished (i.e., on the day of the appearance of the last pre-emergent nymph stage in the control).
[e]Calculated using the geometric mean of three 96-h LC50 (1062 mg Cl/L) divided by EC20 when available, otherwise by maximum acceptable toxicant concentration.
MATC = maximum acceptable toxicant concentration; WCF = when controls finished; NA = not applicable; NC = not calculated (not statistically possible); EC20 = 20% effect concentration; CI = confidence interval; ACR = acute to chronic ratio.

any other treatment (Table 3). Neither maximum allowable toxicant concentrations nor EC20s could be calculated for any of these 3 endpoints. However, variability for these endpoints was relatively low, with coefficients of variation ranging from a minimum of 5 to a maximum of 17, suggesting that they may be useful endpoints for other contaminants with different modes of action.

With EC20s or maximum allowable toxicant concentrations ranging from 165 mg Cl^-/L to 504 mg Cl^-/L, and a mean 96-h LC50 of 1062 mg Cl^-/L, the acute to chronic ratios for chloride ranged from 2.1 to 6.4 (Table 3).

Nitrate test

Percentage of survival to pre-emergent nymph stage was high for the controls and up to 51 mg N-NO_3/L (Table 4). None of the mayflies in the 101-mg/L and 201-mg/L treatments survived to pre-emergent nymph stage. All of the organisms in the highest treatment were dead by day 16. In contrast to the chloride chronic test in which all mortality in the second highest exposure concentration occurred on day 7 or before, in the nitrate test, all mortality in the 101-mg/L treatment occurred on or after day 20, when organisms in other treatments were reaching the pre-emergent nymph stage (Figure 3B). Percentage of pre-emergent nymph when controls finished was more sensitive than percentage of survival to pre-emergent nymph stage, with a maximum allowable toxicant concentration of 36 mg/L compared with 72 mg/L for the latter. The EC20s could not be calculated for either of the survival endpoints because of inadequate partial effects. Controls reached the pre-emergent nymph stage by day 21 on average, and the 12.8 mg/L and 26 mg/L treatments had similar means for number of days to pre-emergent nymph stage (Table 4). Organisms in the 51-mg/L treatment reached the pre-emergent nymph stage on day 22 on average, and this mean was significantly different from that of the control. Although none of the organisms in the 101-mg N-NO_3/L treatment reached the pre-emergent nymph stage, 1 individual in this treatment was alive until day 31. Percentage of emergence was 60% in the controls but even higher in the 12.8-mg/L and 26-mg/L treatments.

As was true of the chloride chronic test, the sublethal endpoints of pre-egg-laying weight and number of eggs per

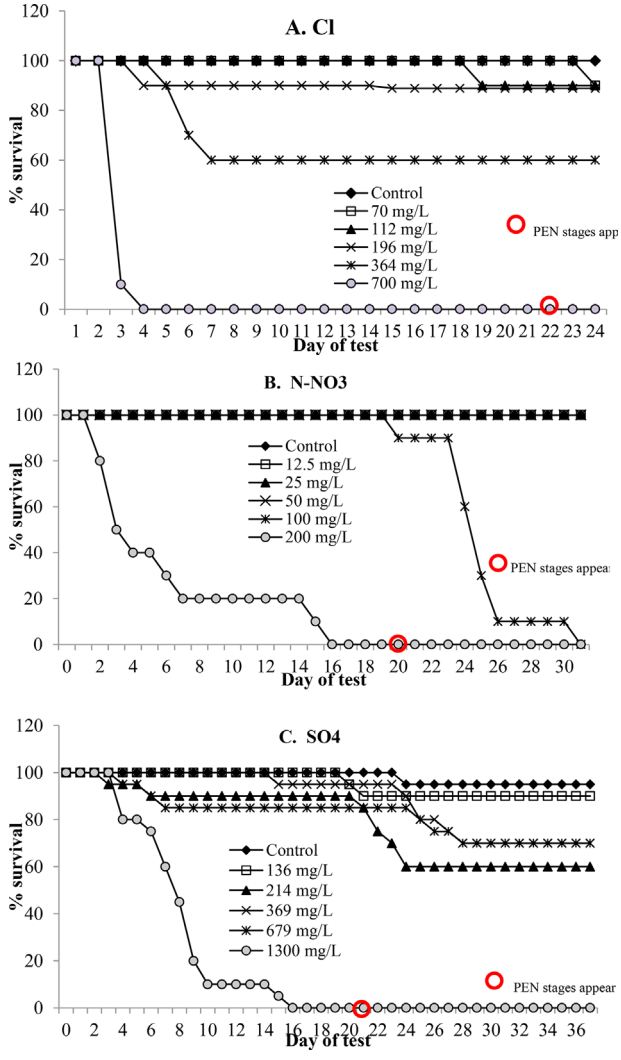

Figure 3. Survival of *Neocloeon triangulifer* larvae to pre-emergent nymph stage at different (**A**) sodium chloride, (**B**) sodium nitrate, and (**C**) sodium sulfate concentrations. Nominal treatment concentrations are shown in legend. Measured values are presented in Tables 4 and 5, respectively.

Table 4. Chronic nitrate (as $NaNO_3$) toxicity data for the mayfly *Neocloeon triangulifer*[a]

$[N\text{-}NO_3^-]$[b] (mg/L)	Conductivity[c] (μmhos/cm)	% pre-emergent nymph WCF[d]	% survival to pre-emergent nymph stage	No. of days to pre-emergent nymph	% emergence	Pre-egg laying weight (mg)	No. of eggs per female	No. of eggs per original female
0.03	374 ± 23	100 A	100 A	21.3 ± 0.6 A	60 A	3.030 ± 0.278 A	1395 ± 244 A	837
12.8	481 ± 17	100 A	100 A	20.7 ± 0.6 A	90 A	3.263 ± 0.179 A	1647 ± 117 A	1483
26	593 ± 22	100 A	100 A	20.4 ± 0.6 A	66 A	3.232 ± 0.103 A	1500 ± 202 A	1000
51	804 ± 24	60 B	100 A	22.4 ± 1.1 B	40 A	3.283 ± 0.239 A	1614 ± 144 A	646
101	1209 ± 30	0 B	0 B	NA	0 B	NA	NA	0
201	2042 ± 73	0 B	0 B	NA	0 B	NA	NA	0
MATC (mg $N\text{-}NO_3$/L)		36	72	36	72	NC	NC	NC
EC20 (mg $N\text{-}NO_3$/L)		NC	NC	NC	39	NC	NC	35
EC20 95% CI		NC	NC	NC	9.2–69	NC	NC	14–85
ACR[e]		5.0	2.5	5.0	4.6	NC	NC	5.1

[a]Within endpoint columns, means followed by different capital letters are significantly different ($p < 0.05$).
[b]Mean measured $N\text{-}NO_3^-$ concentrations are shown.
[c]Conductivity values shown are means (±Standard deviation) of all measurements for the duration of the test.
[d]WCF = when controls finished (i.e., on the day of the appearance of the last pre-emergent nymph stage in the control).
[e]Calculated using 96-h LC50 (179 mg $N\text{-}NO_3$/L) divided by EC20 when available, otherwise by maximum acceptable toxicant concentration.
MATC = maximum acceptable toxicant concentration; NA = not applicable; NC = not calculated (not statistically possible); EC20 = 20% effect concentration; CI = confidence interval; ACR = acute to chronic ratio.

female were not as sensitive as survival or number of days to pre-emergent nymph stage (Table 4). In fact, mean weights and number of eggs for the surviving individuals in the nitrate-exposed treatments were nominally higher than those in the control. We were, however, able to calculate an EC20 for number of eggs per original female of 35 mg $N\text{-}NO_3$/L, which was the lowest chronic value obtained. Variability for pre-egg-laying weight and number of eggs per female were relatively low, with coefficients of variation ranging from 3 to 17.

With EC20s or maximum allowable toxicant concentrations ranging from 35 mg $N\text{-}NO_3$/L to 72 mg $N\text{-}NO_3$/L, and a 96-h LC50 of 179 mg $N\text{-}NO_3$/L, the acute-to-chronic ratios ranged from 2.5 to 5.1 for nitrate (Table 4).

Sulfate test

Percentage of survival to pre-emergent nymph stage was high for the controls and the 129 mg SO_4/L treatment, but was significantly reduced at 209 mg/L and above (Table 5). None of the mayflies in the 1277-mg/L treatments survived to pre-emergent nymph stage. As was the case for the nitrate test, all of the organisms in the highest treatment were dead by day 16 (Figure 3C). For the other treatments that had 60% to 70% survival to pre-emergent nymph stage, most of the mortality occurred after day 21, similar to what was observed in the nitrate test. Percentage of pre-emergent nymph when controls finished was again more sensitive than percentage of survival to pre-emergent nymph stage. Both endpoints had the same maximum allowable toxicant concentration (164 mg/L), but the EC20 for percentage of pre-emergent nymph when controls finished (170 mg/L) was substantially lower than that of the percentage of survival to pre-emergent nymph stage (289 mg/L). Controls reached the pre-emergent nymph stage by day 23 on average, and 2 treatments had significant delays in development, with the 359-mg/L treatment averaging 26 d and the 661-mg/L treatment averaging 32 d (Table 5). None of the organisms in the 1277-mg/L treatment reached the pre-emergent nymph stage. Percentage of emergence was 70% in the controls and roughly decreased with increasing sulfate dose, resulting in an EC20 of 145 mg/L (Table 5).

Again, the sublethal endpoints of pre-egg-laying weight and number of eggs were not as sensitive as survival or number of

Table 5. Chronic sulfate (as Na_2SO_4) toxicity data for the mayfly *Neocloeon triangulifer*[a]

$[SO_4^{2-}]$[b] (mg/L)	Conductivity[c] (μmhos/cm)	% pre-emergent nymph WCF[d]	% survival to pre-emergent nymph stage	No. of days to pre-emergent nymph	% emergence	Pre-egg laying weight (mg)	No. of eggs per female	No. of eggs per original female
57	371 ± 12	95 A	95 A	23.0 ± 1.0 A	70 A	2.550 ± 0.282 A	1124 ± 148 A	749
129	540 ± 10	85 A	90 A	23.5 ± 1.3 A	45 A	2.428 ± 0.410 A	1060 ± 220 A	446
209	715 ± 9	60 B	60 B	23.4 ± 1.1 A	55 A	2.624 ± 0.383 A	1177 ± 209 A	648
359	1057 ± 11	40 B	70 B	26.1 ± 2.1 B	30 B	2.629 ± 0.229 A	1141 ± 130 A	342
661	1719 ± 17	0 B	70 B	32.2 ± 1.7 B	30 B	3.219 ± 0.367 B	1354 ± 150 A	406
1277	3003 ± 39	0 B	0 B	NA	0 B	NA	NA	0
MATC (mg SO_4/L)		164	164	274	274	NC	NC	NC
EC20 (mg SO_4/L)		170	289	528	145	NC	NC	281
EC20 95% CI		128–226	139–437	492–566	69–305	NC	NC	70–1138
ACR[e]		7.2	4.2	2.3	8.5	NC	NC	4.4

[a]Within endpoint columns, means followed by different capital letters are significantly different ($p < 0.05$).
[b]Mean measured SO_4^{2-} concentrations are shown.
[c]Conductivity values shown are means (±Standard deviation) of all measurements for the duration of the test.
[d]WCF = when controls finished (i.e., on the day of the appearance of the last pre-emergent nymph stage in the control).
[e]Calculated using 96-h LC50 (1227 mg SO_4/L) divided by EC20 when available, otherwise by MATC.
MATC = maximum acceptable toxicant concentration; NA = not applicable; NC = not calculated (not statistically possible or valid); EC20 = 20% effect concentration; CI = confidence interval; ACR = acute to chronic ratio.

days to pre-emergent nymph stage (Table 5), but we were able to calculate an EC20 for number of eggs per original female of 281 mg SO_4/L. Mean weights for the surviving individuals in the 661-mg/L treatment were significantly higher than those in the control, likely because of the longer development time. Again, variability for pre-egg-laying weight and number of eggs per female were relatively low, with coefficients of variation ranging from 9 to 21.

With EC20s ranging from 145 SO_4/L to 528 mg SO_4/L, and a 96-h LC50 of 1227 mg SO_4/L, the acute-to-chronic ratios ranged from 2.3 to 8.5 for sulfate (Table 5).

Relative sensitivity of endpoints

We were able to generate at least 4 acute-to-chronic ratios for each of the 3 chronic tests conducted (Table 6). To assess the relative sensitivity of the various endpoints over the 3 tests, we calculated relative acute-to-chronic ratios by dividing each individual acute-to-chronic ratio by the highest acute-to-chronic ratio for each particular test, thereby creating a ranking with a maximum of 1.00 (highest acute-to-chronic ratio) and a minimum of 0 (no acute-to-chronic ratio calculated). By taking the average relative acute-to-chronic ratio for each endpoint, we determined that percentage of pre-emergent nymph when controls finished was the most sensitive endpoint across the 3 tests, with an average relative acute-to-chronic ratio of 0.94, and pre-egg-laying weight and number of eggs per female were the least sensitive, with no acute-to-chronic ratios calculated (Table 6).

DISCUSSION

Our mean 96-h chloride LC50 of 1062 mg Cl/L at 25 °C and a hardness of 93 mg/L was higher than the 48-h LC50 of 399 mg Cl/L (calculated from the reported value for NaCl) reported by Struewing et al. [15] at the same temperature and a similar hardness. However, in the same paper, Struewing et al. [15] reported a 14-d LC50 of 505 mg Cl/L, which, being higher than their 48-h LC50, suggests that perhaps their reported acute tests were an overestimate of sensitivity. Based on results of separate experiments conducted in our laboratory (see Supplemental Data, Table S1), possibly this discrepancy is attributable to the feeding method used by the respective laboratories. The Struewing [15] study fed acute tests using a concentrated mixed diatom suspension, whereas the present study used live diatom biofilm scrapings as food. We conducted acute toxicity tests with *N. triangulifer* using both food types (refrigerated mixed diatom suspension and live biofilm scrapings) but otherwise with identical methods, and obtained substantially different LC50 values (~2-fold difference with non-overlapping confidence intervals) in the chloride salt tests (Supplemental Data, Table S1). Interestingly, although a similar difference was observed in tests with potassium chloride, the same comparisons with sulfate and nitrate salts resulted in LC50s that were quite similar between food types (Supplemental Data, Table S1), so this appears to be a phenomenon specific to chloride. At this time we are unable to speculate as to the mechanism for this difference. Our acute chloride toxicity results lie between those previously mentioned [15] and the findings of the Stroud Water Research Center (J. Jackson, Stroud Water Research Center, Avondale, PA, USA, unpublished data), who generated 48-h LC50s in tests conducted at 20 °C of 1423 mg Cl/L at a hardness of 22 to 42 (Dyberry Creek dilution water), and 2459 mg Cl/L at a hardness of approximately 100 (White Clay Creek dilution water), using naturally colonized live algal biofilm plates as food. With longer duration and a higher test temperature, believing that the Stroud Water Research Center results would be similar to ours is reasonable. All of these described tests were conducted with first instar larvae.

Our full-life chronic toxicity values for chloride, EC20s of 165 mg Cl/L to 190 mg Cl/L, and maximum allowable toxicant concentrations of 265 Cl/L to 504 mg Cl/L are similar to 14-d 25% inhibitory concentrations (IC25s) reported by Struewing et al. [15] in water with a similar hardness (~90 mg/L). Their values ranged from 139 Cl/L to 224 mg Cl/L (calculated from reported NaCl IC25s). Their acute-to-chronic ratios ranged from 0.79 to 2.9, whereas ours ranged from 2.1 to 6.4, a function of our higher LC50. Although they found weight and body length to be sensitive endpoints, our survival and development time data were more sensitive to chloride. A potential explanation for this different finding is that our weights were for egg-bearing adults that successfully emerged, whereas Struewing et al. [15] measured weight on approximately 14-d-old nymphs. Because we observed developmental delays (greater mean number of days to reach pre-emergent nymph stage at 362 mg Cl/L compared with lower treatments), we likely would have obtained similar results regarding weight if we ended the test at 14 d. Comparing endpoints is difficult because of the disparity in test duration, but another difference in the 2 test systems is that, again, Struewing et al. [15] fed their organisms a loose suspension of diatoms, whereas we fed ours live diatom biofilm scrapings. In our preliminary experiments, we were unable to bring mayflies to adulthood by using a refrigerated loose diatom suspension, even when plenty of excess food was available.

Table 6. Actual and relative acute to chronic ratios generated in the present study for three chronic toxicity tests with sodium salts[a]

	C1 test		$N\text{-}NO_3$ test		SO_4 test		
Endpoint	ACR	Relative ACR	ACR	Relative ACR	ACR	Relative ACR	Average relative ACR
% pre-emergent nymph WCF[b]	6.4	1.00	5.0	0.98	7.2	0.85	0.94
% survival to pre-emergent nymph stage	5.6	0.88	2.5	0.49	4.2	0.49	0.62
No. of days to pre-emergent nymph	4.0	0.63	5.0	0.98	2.3	0.27	0.63
% emergence	2.1	0.33	4.6	0.90	8.5	1.00	0.74
Weight[c]	NC	0	NC	0	NC	0	0
No. of eggs/female	NC	0	NC	0	NC	0	0
No. of eggs/original female	NC	0	5.1	1.00	4.4	0.52	0.51

[a]Relative acute-to-chronic ratios were calculated by dividing each individual acute-to-chronic ratio for a given test by the highest acute-to-chronic ratio for that test. A high relative acute-to-chronic ratio indicates high sensitivity (i.e., a larger difference between the chronic endpoint and the 96-h LC50).
[b]WCF = when controls finished (i.e., when the last control individual has reached pre-emergent nymph stage).
[c]Weight = pre-egg-laying live weight of adults.
ACR = acute to chronic ratio; WCF = when controls finished; NC = not calculated; LC50 = median lethal concentration.

The Stroud Water Research Center (J. Jackson, Stroud Water Research Center, Avondale, PA, USA, unpublished data) reported full-life chronic chloride toxicity to *N. triangulifer,* using naturally colonized periphyton plates as food. Their most sensitive endpoints were development time and instantaneous growth rate. Despite their testing at 20 °C, their maximum allowable toxicant concentrations in water with a similar hardness to ours (White Clay Creek) ranged from 177 Cl/L to 708 mg Cl/L. Thus, our full-life chronic method using laboratory-cultured food appears to produce chronic values similar to those produced using natural food sources.

In other chronic toxicity studies using this species or closely related mayflies with chloride dominated salts, Hassell et al. [16] obtained 21-d conductivity LC50s of 890 μS/cm to 2700 μS/cm in 2 different dilution waters at 15 °C, using wild-caught *Centroptilum* sp., and Johnson et al. [18] reported an EC20 of 672 μS/cm at 24.5 °C in a mesocosm study with *N. triangulifer,* using a combination of $CaCl_2$ and NaCl. Both of these tests were conducted with natural foods: conditioned leaves for the former and naturally colonizing periphyton for the latter. In our chloride test, we calculated a conductivity EC20 of 895 μS/cm (709–1129 μS/cm) for the most sensitive endpoint, so again, our test with cultured food can produce similar results to those with natural foods.

Other mayfly genera have been investigated for sodium chloride sensitivity in subchronic tests. For example, Diamond et al. [24] tested *Stenonema modestum* (Heptageniidae) at 12 °C in 14-d exposures. The maximum allowable toxicant concentrations for survival and molting endpoints ranged from 1410 mg Cl/L to 3798 mg Cl/L (calculated from salt concentrations). Echols et al. [3] observed a mean *Isonychia bicolor* 7-d NOEC of 855 mg Cl/L at 20 °C to 23 °C. Goetsch and Palmer [25] reported a 96-h LC50 between 1500 mg Cl/L and 2500 mg Cl/L for *Tricorythus* sp. in an unfed test at 10 °C to 16 °C. K. Allan recorded a 72-h conductivity LC50 of 12 600 μS/cm for the leptophlebiid mayfly *Nousia* sp. using sodium and chloride–dominated saline waters. A 7-d acclimation to higher salinity water did not significantly alter conductivity LC50s for that species (K. Allan, 2006, Master's thesis, University of Tasmania, Hobart, TAS, Australia). Although these effect levels are higher than those observed in our NaCl chronic test, comparisons are tenuous because of the differences in test temperatures, duration, endpoints, and the fact that all of these studies used wild-collected organisms.

Much less information is available in the literature on chronic toxicity of sulfate salts to *N. triangulifer* and related species. Kunz et al. [12] conducted static, nonrenewal exposures of first instar nymphs through adult emergence (~35 d) at ambient laboratory temperatures of 21 °C, using naturally colonized periphyton plates (from Stroud Water Research Center) as food and survival (emergence) and biomass as endpoints. They developed reconstituted waters to simulate major ion compositions typical of streams impacted by coal mining in Appalachian streams. One of the waters tested ("Upper Dempsey") was dominated by Na and SO_4, but in contrast to our findings, it did not cause significant toxicity at the highest test concentration (~640 mg SO_4/L). We observed EC20s ranging from 145 SO_4/L to 528 mg SO_4/L, but our test was conducted at a hardness of 95 mg/L, whereas the Kunz et al. [12] test was conducted at a hardness of 220 mg/L. Hardness has been shown to have a strong influence on sodium sulfate toxicity to other species [26,27]. In 2 other reconstituted waters having an ionic composition dominated by Ca, Mg, HCO_3, and SO_4, Kunz et al. [12] did observe significant toxicity to *N. triangulifer* at sulfate concentrations ranging from 386 mg/L ("Winding Shoals" water) to approximately 770 mg/L ("Boardtree" water; sulfate not measured). In terms of conductivity, the Winding Shoals and Boardtree waters were toxic to *N. triangulifer* at approximately 800 μS/cm and 1300 μS/cm, respectively [12]. We calculated a conductivity EC20 for our sodium sulfate test of 725 μS/cm.

Goetsch and Palmer [25] reported a 96-h LC50 of 532 mg SO_4/L for the mayfly *Tricorythus* sp. in sodium sulfate exposures at approximately 10 °C to 15 °C. This is substantially lower than our 96-h LC50 of 1227 mg SO_4/L, but in addition to using a different species with potentially different sensitivity to sulfate, the *Tricorythus* test was conducted at a moderately lower hardness (~70 mg/L as $CaCO_3$), and the test was not fed. Conversely, our conductivity EC20 (725 μS/cm) for *N. triangulifer* is quite similar to 7-d maximum allowable toxicant concentrations reported by Kennedy et al. [28] (737–773 μS/cm) for *Isonychia bicolor* exposed to a simulated mine effluent dominated by sodium and sulfate.

To our knowledge, these are the first acute and chronic nitrate toxicity values reported for *N. triangulifer*, but our lowest maximum allowable toxicant concentrations (36 mg N-NO_3/L) and EC20 (35 mg N-NO_3/L) are strikingly similar to a 20-d threshold effect concentration (equivalent to an maximum allowable toxicant concentration) reported for the New Zealand leptophlebiid *Deleatidium* sp. [29]. Our 96-h LC50 for *N. triangulifer* (179 mg N-NO_3/L) would place this species fifth in sensitivity among the invertebrate data previously compiled [30]. Our lowest chronic value is similar but nominally less sensitive than the mean maximum allowable toxicant concentration (22 mg N-NO_3/L, calculated from NOEC and least observable effect concentration values) reported for the water flea *Ceriodaphnia dubia* [31], but it is substantially more sensitive than *Daphnia magna* (mean maximum allowable toxicant concentration = 507 mg N-NO_3/L [31]), and the apple snail *Pomacea paludosa* (mean 14-d EC50 = 560 mg N-NO_3/L) [32].

For each of our 3 full-life chronic toxicity tests, we evaluated 7 endpoints: 2 involving survival to pre-emergent nymph stage, 1 involving development time, 1 involving adult emergence rates, and 3 involving adult weight or fecundity. Based on average relative acute-to-chronic ratio (Table 6), the most sensitive endpoints in our tests were those involved with survival, emergence, and development time. This suggests that exposure to sublethal concentrations of these sodium salts in the field may not affect somatic growth or fecundity directly, but could have phenological effects that may indirectly impact population growth. The weight and number of eggs per female endpoints were not sensitive in any of our 3 tests; however, others, using different testing scenarios, have found growth and biomass endpoints to be sensitive ([12,15,18]; J. Jackson, Stroud Water Research Center, Avondale, PA, USA, unpublished data). As stated previously, this disparity can in part be attributed to differences in when tests are ended, for example, based on our development time results (number of days to pre-emergent nymph stage), had we ended our tests at day 14 as in Struewing et al. [15], we likely would have seen differences among treatments in organism weights. Furthermore, we believe that even in a test conducted according to the method in our present study, weight and fecundity endpoints should be evaluated because chemicals with different modes of action than those tested in the present study may cause different types of effects.

In our chloride chronic test, nearly all mortality occurred by day 7, whereas in the nitrate chronic, mortality in the highest concentration occurred early in the test (beginning on day 2), but in the second highest concentration, no mortality occurred until pre-emergent nymph stages were appearing in other treatments (day 20; Figure 3). A similar pattern was observed in the sodium sulfate chronic. This suggests that the most sensitive life stage may be dependent on the contaminant, but it also supports the findings of others [19,33], who in field and laboratory studies exposing *N. triangulifer* to metals found that metamorphosis to the imago was a highly sensitive life-stage.

Based on our starvation test, acceptable control survival (≥90%) appears to not be possible in an unfed 48-h or 96-h acute toxicity test conducted at 25 °C. Similar results were reported for this species by Struewing et al. [15], who had 11% survival after 48-h in USEPA moderately hard reconstituted water with no food, and by the Stroud Water Research Center (J. Jackson, Stroud Water Research Center, Avondale, PA, USA, unpublished data), who in 4 of 5 waters tested had approximately 10% survival or less after 48 h. The ASTM International [4] method for conducting acute toxicity tests states that organisms should not be fed during acute toxicity tests, although it makes an exception for mysid shrimp, which are severely stressed if not fed. In the present study, the nature of the chemicals tested is such that the presence of food would not be expected to alter bioavailability of the contaminants, but the need to feed *N. triangulifer* in acute tests will be a factor to consider in tests with other contaminant types. Poteat and Buchwalter [34] contend that in the case of metals in particular, dietary uptake is a more ecologically valid measurement for aquatic insects than dissolved exposures, but in general, water quality criteria development still uses data from waterborne exposures, so this is an issue that may require some flexibility.

We compared the relationship between pre-egg-laying weight and the number of eggs produced per female in the present study with that reported in Weaver et al. [14], and although our fecundity numbers were consistently lower than those predicted by their equation (Figure 4), the slopes of the lines were quite similar whether we used only control data (Figure 4A) or both control and exposure data (Figure 4B). If we use our data to predict a minimum adult weight at which 1000 eggs or more would be produced in the same manner as that described in Weaver et al. [14], we obtain values of 2.95 mg for control-only data, and 2.72 for control plus exposure data. These numbers bracket the previously reported value of 2.8 mg [14]. In other words, using our "controls only" equation to predict number of eggs based on a weight of 2.0 mg results in a value that is 80% of the result using the Weaver et al. [14] equation, and at a weight of 3.8 mg (just above our maximum), our equation produced a number of eggs value that was 94% of that using the Weaver et al. [14] equation. These slight differences may be attributable to acclimation to different waters or different sources of within-laboratory error, but the overall similarity of these relationships obtained using similar methods of producing laboratory-cultured food indicates good potential for standardization.

In the process of adapting the Weaver et al. [14] method and developing our own full-life chronic toxicity method, a few of the lessons we learned are as follows. First, for culturing, we have obtained best results when seeding stocks and cages for diatom slides with fresh diatom stocks that have never been refrigerated. Second, further work should be done to specifically characterize ion and nutrient concentrations required for diatom slide and diatom stock media. We currently use dechlorinated tap water with an added nutrient and trace metal solution to culture diatom stocks and slides. Although we have analyzed tap water samples for ionic composition, this is likely variable over time because of the nature of municipal water supplies. One of the most variable aspects of the current method remains the quality of diatom slides, and better knowledge of specific ion requirements for diatoms will help to reduce this variability. Third, our results suggest that mayflies require live biofilms for robust development to adulthood. In numerous attempts, feeding concentrated mixed diatom suspensions that have been refrigerated did not result in development to pre-emergent nymph stage. Fourth, before feeding live biofilms to mayflies, a sample of the biofilm slide must be examined under a compound microscope to ensure that most of the cells are intact, and that the biofilm is not made up of amorphous material. Color of the biofilm does not appear to be a good predictor of food quality. Finally, having a higher percentage of emergence in controls during chronic toxicity tests would be desirable. One current thought is that because the lowest nitrate concentration had 90% emergence during that test, perhaps the addition of a low concentration of nitrate to culture medium and test dilution water would improve the food quality and therefore increase the rate of emergence.

Mayflies have received much attention recently because of their apparent sensitivity to mining influences in Appalachian headwater streams, with declines attributed to elevated conductivity, a surrogate measure of major ion concentrations of total dissolved solids [35–38]. Until recently, a disconnect

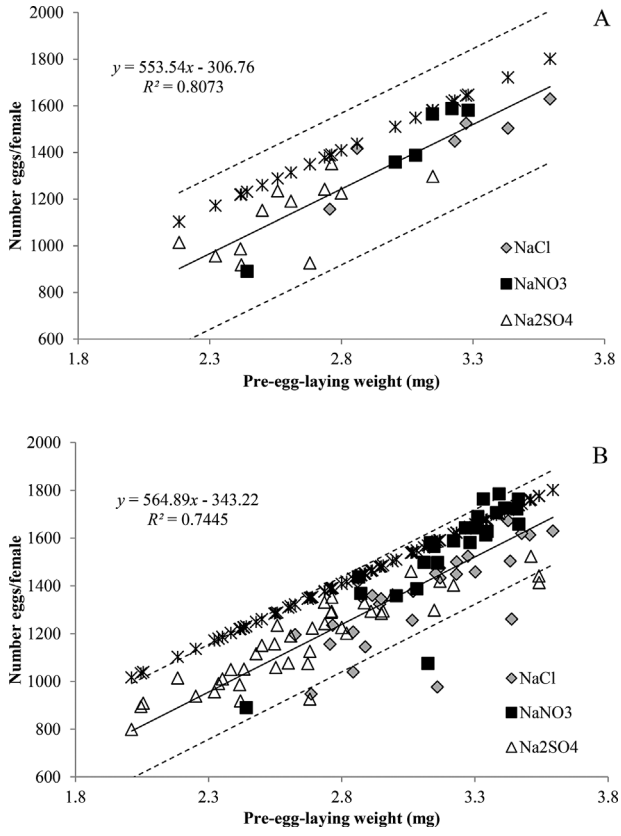

Figure 4. Relationship between pre–egg-laying wet weight of *Neocloeon triangulifer* and number of eggs produced in (**A**) controls only and (**B**) all treatments of 3 chronic toxicity tests. Asterisks are number of eggs/female predicted by the equation from Weaver et al. [1] based on our mayfly weights. Dashed lines show 95% confidence intervals for the solid line described by the equation in each panel.

has been perceived between what major ion concentrations are toxic to organisms in the laboratory versus what concentrations cause impairment to endemic benthic macroinvertebrate communities. Although no laboratory exposure system can completely replicate exposure scenarios experienced by organisms in situ, our observation of effects at various life-stages and documentation of potential phenological impacts brings some environmental relevance in addition to the fact that this is an organism that better represents benthic macroinvertebrate communities than standard crustacean models. We believe that the continued use of this sensitive mayfly species in laboratory studies will help to close this gap in understanding.

SUPPLEMENTAL DATA

Table S1. (15 KB DOC).

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Data availability—Contact David Soucek at soucek@illinois.edu for available data.

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